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Bridgewater State University

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ABSTRACT

Over the past few years, honeybee populations (*Apis mellifera*) have drastically decreased due to Colony Collapse Disorder (CCD). CCD occurs when most of the honeybees abandon their hive and queen, and do not return. Around the same time CCD became commonly identified, commercial beekeepers switched the bee's food from sucrose to a less expensive, but more processed substitute of high fructose corn syrup (HFCS), suggesting there might be a relationship between the types of food and CCD. Therefore, the goal of this project is to identify changes in carbohydrate metabolism due to HFCS. To determine these changes, gene expression was measured within the glycolysis and gluconeogenesis pathways. For seven days, bees from 9 hives were fed honey, high fructose corn syrup (HFCS), corn syrup (CS), and sucrose. After termination, mRNA was extracted and cDNA synthesized for Quantitative PCR (qPCR) analysis with SYBR green to detect changes in expression for 14 genes in these pathways. In the glycolysis pathway, the enzymes glucokinase (GK1), phosphofructokinase (PFK), triose phosphate isomerase (TPS1), pyruvate kinase (PK2) and lactate dehydrogenase (LDHA) were down-regulated in HFCS compared to honey. Further, when bees were fed CS, they down-regulated most of the genes that are down-regulated in HFCS. However, these genes were not changed when bees were fed sucrose. For genes in both glycolysis and gluconeogenesis, gene expression did not change when given HFCS. This was also observed for genes in just gluconeogenesis. In summary, HFCS and CS diets both lead to down-regulated genes specific to the glycolysis pathway when compared to honey.

INTRODUCTION

The Honeybee Life Cycle

Life cycle stages are common across different organisms. The honeybee is one of many organisms that separates its life cycle into prominent stages. In the honeybee life cycle, all bees must go through four phases known as the egg, larva, pupa, and adult stage (Winston, 1987).

The egg stage is the first phase of the honeybee life cycle. Eggs are white in color, cylindrical and an elongated oval shape. One at a time, eggs are laid by the queen bee into specialized honeycomb cells known as worker or drone cells. Only one egg is laid per cell, where they are attached to the floor by the queen. There are two types of eggs that can be laid, fertilized and unfertilized. Fertilized eggs develop into worker bees or queen bees; and unfertilized egg develop into drones. The larval stage is the second stage in honeybee development. Honeybee larvae are a worm-like grubs that are white in color. The larvae do not have legs, antennae, wings, segmented bodies, or a stinger. Larvae are fed by worker bees, and go through 5 molts during the entire larval stage. On the last days of the larval stage, the cell is capped and the larva spends the rest of larval life preparing for pupation, known as the pre-pupal stage (Winston, 1987). Inside the capped cell, the larva will stretch and uncurl, excreting silk from their soon-to-be thoracic salivary glands. The larval stage is completed with the final molt and metamorphosis into pupae (Figure 1).

In the pupae stage, the head, antennae, mouthparts, legs and abdomen begin to form. The wings remain underdeveloped and small, not fully evolving until the adult stage. As the pupal stage continues, the cuticle becomes darker, and intense color changes occur. Internally, the muscles and organ systems of the bee develop to be more complex and change dramatically. When the bee sheds its exoskeleton, it enters the adult stage (Figure 1). After the shedding of exoskeleton there is an emergence period. It emergences by pushing and chewing through the capped cell with its mandibles. Once fully emerged it spreads its wings, unfolds its antennae and waits for the hairs on its body to dry (Winston, 1987).

The adult stage officially starts after emergence. The cuticle hardens within 12-24 hours, and a few days are dedicated to glandular development, organ development, and fat growth (Winston, 1987). This requires proper nutrition and pollen consumption for protein for all castes. Insufficient pollen consumption early in life results in poor glandular development and shorter life span (Haydak, 1970; Maurizio, 1950). Workers begin consuming pollen only a couple hours after emergence, and are unable to sting due to under developed exoskeleton around the sting glands. Full development takes approximately 8-10 days (Winston, 1987).

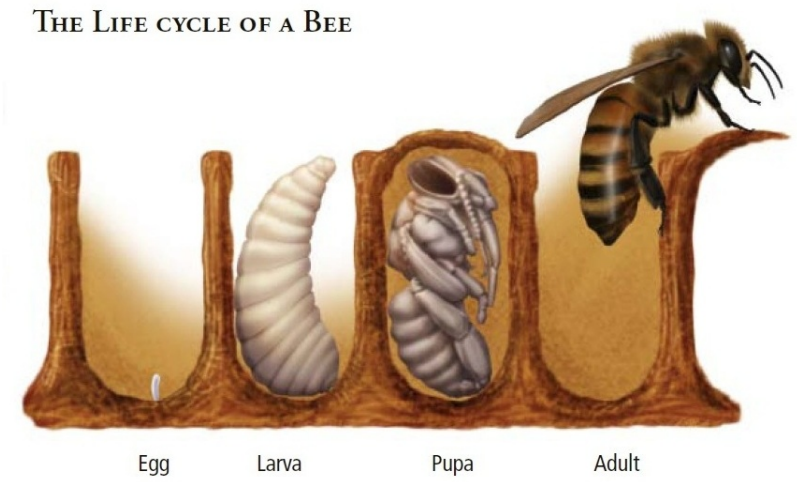


Figure 1: Honeybee Life cycle. (Figure adapted from: NASA, 2017)

Food

Food type is crucial to bee maturation and development. Each caste has different nutritional needs and methods of feeding that satisfy certain food requirements. Food can be specific to a certain stage or caste, but the starting components are always the same. The two starting components are nectar and pollen, floral products that are required for all stages of the life cycle from larval to adult development. Nectar is a plant produced sugar that primarily comes from flowers. Its main function is to provide carbohydrates and energy for the bees. Floral nectar can be 5 to 80% sugar, and can also contain minerals, vitamins, organics acids, lipids, nitrogenous compounds, aromatic substances and pigments. These others constituents exist in small quantities and differ by strain of nectar. The main sugars are glucose, fructose and sucrose (Winston, 1987).

While nectar is filled with carbohydrates, pollen consists of the proteins, lipids, vitamins and minerals. While all components of pollen form the foundation for multiple biological functions, the most important component for bees is the protein. Depending on the type, pollen can range from 6 to 28% protein content, and is the only natural protein source for bees. Pollen also contains lipids, ranging from 1 to 20%, and other materials like sterols, starches, vitamins and minerals composing less than 0.5% each (Winston, 1987). When foragers bring back pollen they initially pack it into honey comb cells to perform a series of steps which prevent any pollen development and increase longevity for storage. Pollen digestion occurs in the comb cells, and can be accomplished in two ways. One way it can be initiated is by bacterial action. Bacterial activity is normally suppressed by a phytocidal acid secreted by the hypopharyngeal or mandibular glands of workers, but when enzymes excreted by workers mix with honey some bacterial action occurs (Maurizio, 1959; Chauvin, and Lavin, 1956; Lavie, 1956; Pain and Maugenet, 1966). Although fermentation and bacteria action could be beneficial to pollen longevity, the phytocidal acid is added to permanently stop germination and bacteria growth (Winston, 1987). When pollen is completely processed, it is commonly referred to as “bee bread”, due to its ability to be eaten at any time.

The second way pollen can be digested is directly within the bee. When pollen is ingested, there is minimal mechanical breakdown involved. This may cause complications for both larvae and adults due to the hard, indigestible cell wall components. The pollen bolus passes the honey stomach into the midgut where digestion occurs. Enzyme secreting membranes tighten around the

bolus and protect the soft tissue from sharp edges of the pollen wall (Barker and Lehner, 1972). The usable nutrients are absorbed and pass through the digestive system within 1-3 hours. The process is similar in both larvae and adult bees, although larvae do not ingest as much pollen (Winston, 1987).

Nutritional Effects on Caste Determination

Nutrition is not just important to keeping honeybee populations fed and thriving, it also plays a role in caste determination. Caste determination regulates the structure and efficiency of a honeybee colony, and is affected by two things: nutrition and hormonal influence.

Nutrition affects the caste system by determining which caste a larva will enter. Early nutrition for larva consist of two main substances, each made directly by bees: brood food and royal jelly. Brood food precursors consist of a clear component, that is secreted by the hypopharyngeal glands, and a milky-white component, that is a mixture of some hypopharyngeal, as well as mandibular gland secretions. The composition of mandibular gland secretions consists primarily of 10-hydroxyl-2-heptanone, the main lipid found in larval food (Butenandt and Rembold, 1957), and 10-hydroxy-2-decenoic acid. Hypopharyngeal gland secretions are composed of proteins, some lipids, and vitamins (Patel et al., 1960) as well as the enzyme invertase. These two essential components are mixed with honey, water, and some digestive enzymes to yield brood food, which is stored in honeycomb cells and can be readily fed to developing larvae.

For worker larva, the primary food source is brood food. During the first 2 days of larval life, the brood food consists of 20-40% white component and 60-80% clear component (Winston, 1987). Starting on the third day, the proportion of white component decreases, leaving mostly clear component and the other ingredients discussed above (Shuel and Dixon, 1959; Patel et al., 1960). At this time, a little pollen is also introduced. Nurse bees provide a little more pollen on the fourth day, with the most being administered on the fifth day of larval life (Morton, 1950; Jung-Hoffmann, 1966; Matsuka et al., 1973)

Once a worker larva enters adulthood, its diet changes significantly. Adult workers follow the basics of honey and pollen, with some brood food incorporated through food exchange (Winston, 1987). Honey provides the appropriate amount of energy needed to support survival and perform many different tasks. The more active a worker is, the more sugar it needs to maintain proper energy levels. Workers eat honey by either lapping it from comb cells or receiving it through regurgitation from other workers. Pollen is not used as an energy source for bees. Instead, pollen is ingested for protein and used to support glandular development in the first 8-10 days of adult life (Winston, 1987). Lack of pollen in this time frame has resulted in shorter life spans of workers, and poor development of the hypopharyngeal glands and fat bodies (Haydak, 1937a, b; Maurizio, 1950; Groot, 1951; Weiss, 1984). After 10 days of adult life, workers do not need pollen unless they are producing brood food to feed to larvae.

Workers and queens both come from fertilized eggs, transitioning into the larval stage at the same time. The larvae are totipotent, or capable of developing into either a worker or queen when under the correct conditions. Nutrient quality and quantity are two factors that helps determine which path a female larva will take. Queen larvae need different nutritional requirements to ensure proper growth and development. Unlikely worker larva, queen larvae are fed royal jelly instead of brood food. Royal jelly has 10 times more panthothenic acid and 18 times more biopterin compared to what is fed to workers (Haydak and Vivino, 1950; Lingens and

Rembold, 1959; Hanser and Rembold, 1960). For the first three days, royal jelly consists of mostly the milky-white component from the mandibular glands, which is higher in concentration than what workers receive. During the last two days of larval life, queens are given a 1:1 ratio of clear to white component (Jung-Hoffman 1967).

The royal jelly is not the only thing that distinguishes larval queens from workers. Queens must also consume more food than workers, especially within the last three days of larval life. The effects of differential feeding are not seen until day three, due to the rapid spike in development queens obtain on the final two days from food intake. Queen larvae are also visited 10 times more frequently then worker larvae by nurse bees (Lindauer, 1952). Nurse bees do not feed specific larvae, but know how to adjust their secretions to the right proportions depending on the type they are tending (Free, 1960a). Once transitioned into adulthood, queens switch their food from royal jelly to mostly brood food with some honey (Allen, 1955, 1960; Haydak, 1970). Workers that produce brood food feed the queen directly through mouth-mouth exchange, unless the queens find themselves isolated (Weiss, 1967). Having proper nutrition supplied to the queen has been correlated to egg laying rate. As feeding increases, egg laying increases and colonies grow (Chauvin, 1956; Allen, 1960).

Worker Tasks Associated with Proper Nutrition

Starting immediately after emergence, workers perform all labor-intensive tasks in the nest. Their communal routine includes long periods of rest intermixed with organized and efficient periods of activity. Although worker tasks cover a wide range of responsibilities like guarding, cleaning and security, there are three major tasks specifically designed for proper nutrition: foraging, food handling, and nursing all ensure that food gets properly acquired and distributed to the hive colony.

The first worker task associated with proper nutrition is foraging. Foragers leave the nest in search of nectar, pollen, and water. Colony conditions determine the need for a particular resource before departure, and can change between foraging flights. On average, foragers make 10 trips per day, although Ribbands (1949) recorded one worker making 29 trips in one day. Nectar is taken back to the hive and may be fed to brood and adults immediately, but is normally converted into honey first. To make honey, bees will regurgitate the nectar into the oral cavity where it is evaporated on the worker's proboscis (tongue). While on the tongue, the hypopharyngeal gland duct behind the tongue opens, releasing the enzyme invertase, which helps process floral nectars into honey (Simpson, 1960; Simpson et al., 1968). It is then placed in a cell and further evaporated by rapidly fanning their wings over it. Other resources acquired on forager flights are brought back and placed in comb cells, where they are then managed by workers called food handlers.

Food handlers package pollen and nectar after receiving it from foragers. Pollen is packed down to the bottom of the cells with the workers' mouth parts, while saliva and honey are used to anchor loose crumbs (Parker, 1926). Packaged pollen is sealed with a thin layer of honey, keeping it preserved for months. Nectar is either regurgitated and lapped up, or taken directly from the forager through mouth-mouth exchange. Typically, 2-3 handlers will take nectar from one forager and bring it to a different part of the nest. There the evaporation of nectar will start through the folding and unfolding of the workers' mouthparts. As the nectar is exposed to air, water gradually evaporates. This process continues for about 20 minutes after which the worker deposits the nectar into a cell. Drying is continued here by fanning until the nectar contains less than 18% water. This

can take up to five days depending on water content, number of workers involved, and amount of nectar being processed (Park, 1925b, 1927, 1928a,b).

Lastly, nurse bees take the stored food and deliver it to brood, larva, and the queens. Nurses are workers with well-developed hypopharyngeal and mandibular glands who are responsible for feeding newly emerged brood and larva. A single larva can be attended to by many nurse bees, who often just visit and inspect more than feed. Lindeur (1952) observed that larvae who were inspected 1926 times within a 72-minute window were only fed 143 times by nurse bees (0.07% of visits). The feeding mechanism starts after a quick cell inspection by placing a drop of food on the bottom of the cell near the occupant's mouth. No direct mouth-mouth food exchange takes place during the feeding process. The nurse bee determines larval age and delivers the correct proportions of white and clear components accordingly.

Honey

Honey is viscous, sweet, and can contain approximately 200 substances (Escuredo, et al. 2013). It is composed primarily of sugar and water, but can also contain small amounts of enzymes, amino acids, minerals, organic acids, vitamins, and aromatic substances (Alqarni et al. 2012). Honey can consist of multiple sugar types including glucose, fructose, sucrose, maltose, and trehalose (Fuente et al., 2011). Monosaccharides such as glucose and fructose equal 75% of the sugar content, while disaccharides such as sucrose make up 10-15%. All of these are responsible for providing high energy values and viscosity (Kamal and Klein, 2011). The other compounds in honey exist in small amounts and varies according to flower species. Protein, organic acid, vitamins, minerals, content is less than 1% (Iglesias et al., 2006; Alqarni and Mahmoud, 2012; Karabagias, et al., 2014). Differences in honey are primarily caused by flower type and geographical region, but can also be caused by the race of bee, climate, and storage time (Escuredo et al., 2014; Tornuk et al., 2013).

Honey is made when foraging worker bee finds nectar. After drinking it, the honey is stored in a forager's "honey stomach." The honey stomach breaks down the nectar to easily digestible forms with enzymes from their hypopharyngeal glands. These enzymes also include a preserving agent to avoid bacteria when the nectar is processed into honey. Once the enzymatic activity is completed in the stomach and mouth and the evaporated process is completed the nectar is considered honey. The honey is the either fed to larvae, adults or stored in a sealed capped cell (Winston, 1987).

High Fructose Corn Syrup (HFCS)

Although honey is the primary food source for adult bees, and is naturally derived from nectar, commercial beekeepers have switched their bee's food from honey to HFCS starting in the 1970s (Barker and Lehner, 1974a, 1978). HFCS is extremely different from honey due to its man-made properties and being derived from corn. There is also a difference between HFCS and regular corn syrup. Corn syrup (CS) is derived from corn and made up of only glucose bound in various chain lengths, while HFCS consists of high amounts of fructose with small amounts of glucose. HFCS is also stable in acidic conditions, like those found in carbonated beverages (White, 2008). Due to its stability and extreme sweetness, HFCS is used in many foods and beverages. The fructose concentration varies resulting in three different formulations known as HFCS 90 (90% fructose, 10% glucose), HFCS 42 (42% fructose, 58% glucose,) and HFCS 55 (55% fructose, 45% glucose;

Table 1). HFCS 90 is made first, and then used to make formulations 42 and 55 (Parker et al., 2010).

Table 1: Percent of Glucose and Fructose found in different sweeteners (Adapted from: White, 2008)

Sugar Type	**HFCS 42**	**HFCS 55**	**Corn Syrup**	**Honey**
Fructose	42%	55%	0%	49%
Glucose	58%	45%	100%	43%

HFCS is made through a multi-step enzymatic hydrolysis of corn starch. The first step is the physical separation of corn components through steeping and softening. This separates the corn starch, corn hull, protein, and oil. Next, corn starch is hydrolyzed into to corn syrup. Amylopectin and amylose are added to the corn starch and heated (Figure 2). Sodium

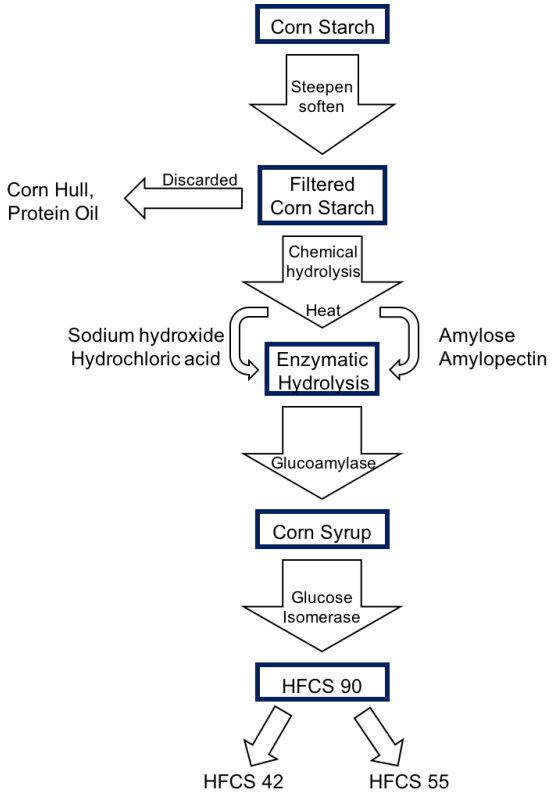


Figure 2: Conversion of Corn Starch into High Fructose Corn Syrup (HFCS). Chemical and enzymatic hydrolysis of corn starch to HFCS. Chemical hydrolysis is performed by amylose and amylopectin, while sodium hydroxide and hydrochloric acid break glucose chains. Glucose isomerase converts most glucose chains into fructose, only leaving a small percent of free floating glucose in the solution. HFCS 90 is diluted with corn syrup to yield formulations 42 and 55.

hydroxide and hydrochloric acid are added, to break the chains of glucose. Once the long glucose chains have been broken, α- amylase hydrolyzes the corn starch into small oligosaccharides, and glucoamylase is added to break the oligosaccharides, resulting in the product of corn syrup

(Figure 2) (Parker et al., 2010). Finally, glucose in the freshly made corn syrup is isomerized by the enzyme glucose isomerase, yielding HFCS 90 (Parker et al., 2010). HFCS 90 is diluted with glucose resulting in HFCS 42 or 55 (Long, 1986; Hanover and White, 1993).

Commercial Use of HFCS

There are multiple reasons why the use of HFCS has become so popular. Known for its sweetness, affordability, solubility and stability, HFCS can be put into any food to ensure longevity of commercial profit. Depending on the formulation, HFCS is sweeter than regular sucrose, and therefore preferable by many commercial buyers. HFCS 55 is sweeter than sucrose, and therefore widely used in juice, soft drinks, and carbonated drinks. HFCS 42 is mildly sweet, and does not mask the natural taste of food. It is used in canned fruits, sauces, soups and also some flavored milks, ice cream, and frozen desserts. Its solubility allows it to stay in solution and its stability allows it to withstand acidic conditions without crystallizing, leaving no need for extra preservatives. HFCS is also used for its affordability at 32 cents/lb. compared to 52 cents/lb. for sucrose (Parker et al., 2010).

Since HFCS is heavily consumed by people, it has been tested for links to certain health conditions such as obesity, diabetes, and cardiovascular diseases in human subjects. Over the past ten years, strong correlations between HFCS consumption and weight gain, obesity, and lipogenesis (Bray et al., 2004). Fructose is absorbed differently than glucose. Glucose is absorbed in the upper intestinal track while fructose is absorbed in the lower intestinal track. This difference, combined and with over-consumption resulted in subjects experiencing weight gain and obesity (Bray et al., 2004). There is also evidence of an effect of HFCS on leptin resistance and insulin resistance, suggesting a link to diabetes. In rats, HFCS 55 promoted changes in hepatic gene expression of lipid metabolism, leading to hepatic lipogenesis (Mock et al., 2017).

HFCS is the top artificial sweetener found in commercial products, and has been linked to multiple health conditions in humans including diabetes, weight gain, obesity, and lipogenesis. Generally, it is fed to other organisms only to help study human diseases, but has recently been used as a substitute for sugar in commercial bee hives. Commercial beekeepers have continued to switch their bee's primary food source from sucrose to HFCS. Honeybees require a constant supply of sugar in order to maintain proper colony health, and HFCS is a cheaper carbohydrate source that is affordable at this high demand. Starting in the 1970s beekeepers made the switch to HFCS (Barker and Lehner, 1974a; 1978) originally using it as a substitute for when resources were scarce. In time and despite the effects of HFCS seen in humans, it became a common food source amongst many beekeepers to promote brood rearing and enhance pollination (Parker et al., 2010).

Introduction to Carbohydrates

When honeybees enter the adult stage, they rely on a strict carbohydrate diet by eating honey as a sugar source for energy. Carbohydrates are classified into three categories: starches, sugars, and fibers. Starches consist of a chain of glucose subunits, and are typically found in vegetables and grains. Sugars are broken down further into two sub-categories known as intrinsic and extrinsic sugars. Intrinsic sugars are found in fruits and milk products, whereas extrinsic sugars are added to food as a sweetener through processing or at the table. Unlike starches, fibers consist of sugar units bound together that cannot be converted into glucose or broken down by digestive enzymes. Instead, it is passed into the large intestine mostly intact and excreted (Slavin and

Carlson, 2014). Carbohydrates are also categorized by structure, which are known as simple sugars and complex sugars. Simple sugars include monosaccharides and disaccharides, and complex sugars include oligosaccharides and polysaccharides. These sugars vary in chain length with monosaccharides being the smallest and polysaccharides being the largest (Figure 3).

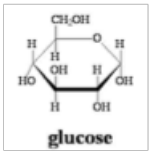
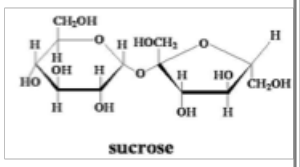
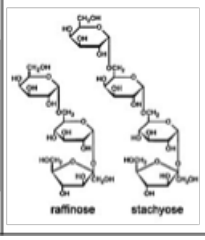
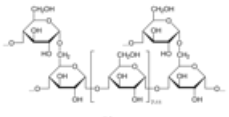
	Simple Sugars		Complex Sugars	
Sugar Type	**Monosaccharides**	**Disaccharides**	**Oligosaccharides**	**Polysaccharides**
Sugar Length	• 1 sugar unit	• 2 sugar units	• 3-10 sugar units	• 10+ sugar units
Examples	• Glucose • Fructose	• Sucrose • Lactose • Maltose	• Raffinose • Stachyose	• Starch • Glycogen
Structure				

Figure 3: Classification of Sugars based of Linkages. Sugars are classified into two main groups known as simple and complex which are further broken down into other sugar types. These sugars are classified by chain length.

Energy Metabolism

Metabolism is the multistep process of breaking down different compounds within an organism. Metabolic pathways are highly-regulated systems, with a series of enzymatic reactions that are primarily irreversible. These enzymatic reactions respond to changing conditions through feedback or feed-forward inhibition, sharing common intermediates. Metabolism can be broken down into two types of pathways, catabolic and anabolic. Catabolism derives energy from fuels which is used for biological purpose. Catabolism is used to break down carbohydrates, proteins, and fats yielding in an energy source. The type of energy source differs depending on the organism, and may include ATP, NADH, $FADH_2$, or Acetyl CoA. Anabolism inputs energy to make new macromolecules. Examples of this processes includes gluconeogenesis, DNA/RNA synthesis, and fatty acid synthesis.

Introduction to Carbohydrate Metabolism

Glycolysis is the process of breaking down glucose for energy. It can also break down other sugars, like fructose, mannose, and galactose (Figure 4), that enter at different parts of the pathway due to different structures. Glucose enters at the beginning on the pathway, whereas galactose enters at glucose-6-phosphate (Figure 5, 8), the second step in glycolysis (Figures 4, 6). Mannose and fructose from muscle enter at fructose-6-phosphate, which is further into glycolysis (Figure 4, 5), while fructose from the liver enters even further at GAPDH (Figure 4, 7). Once broken down, sugar travels as an energy source to cells through the circulatory system and is received at target organs through cellular uptake. Levels in the blood are tightly regulated by insulin that binds to receptors on tissues. Insulin receptors vary depending on placement in the body, and activate glucose intake receptors to supply the sugar to tissues. For example, GLUT4 receptors are found in muscle, GLUT1 receptors are in blood, GLUT2 is in the liver, and GLUT3 is found in the brain.

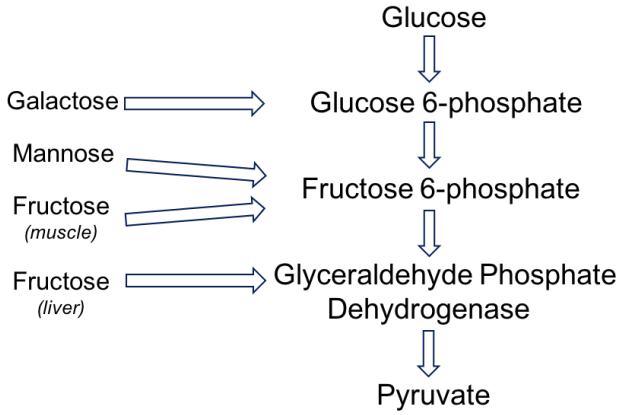


Figure 4: Entry of glucose epimers in glycolysis. Epimers of glucose are depicted entering at different stages within glycolysis due to structural and configuration differences.

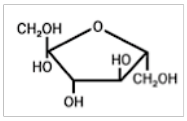
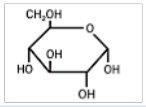
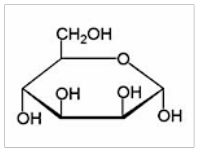
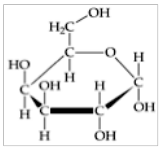
Name	Structure	Ingestion	Absorption	Mechanism
Fructose		• Oral ingestion • Bi-product of sucrose break down	• Absorbed in liver or muscle	• From Muscle: phosphorylated by hexokinase to form fructose-6-phosphate glycolysis intermediate • From Liver: phosphorylated by fructokinase to form fructose-1-phosphate glycolysis intermediate
Glucose		• Oral ingestion • Derived from glycogen or pyruvate	• Absorbed in blood, muscles, and various parts of the body for energy	• Glycolysis pathway yields pyruvate • Enter TCA cycle resulting in high levels of ATP • For muscle: forms lactate
Mannose		• Epimer of glucose • Oral ingestion	• Similar pathway to glucose • Converted into glycolysis pathway, absorbed in same way as glucose.	• Phosphorylated by hexokinase to mannose-6-phosphate • Phosphomannose isomerase isomerizes into fructose 6-phosphate • Glycolysis pathway continues normally
Galactose		• Oral ingestion of lactose	• Absorbed primarily in liver • Similar pathway to glucose	• Synthesized by galactokinase instead of hexokinase to form galactose-1- phosphate • Converted to glucose-1-phosphate by uridylyl transferase • Converted to glucose-6-phosphate by phosphoglucomutase • Continues with glycolysis

Figure 5: Sugars involved in glycolysis. Different sugars regularly broken down through glycolysis. Sugars are followed by method of ingestion and absorption, as well as the molecular mechanisms for integration (Information from: Institute of Medicine, Food, and Nutrition Board, 2005)

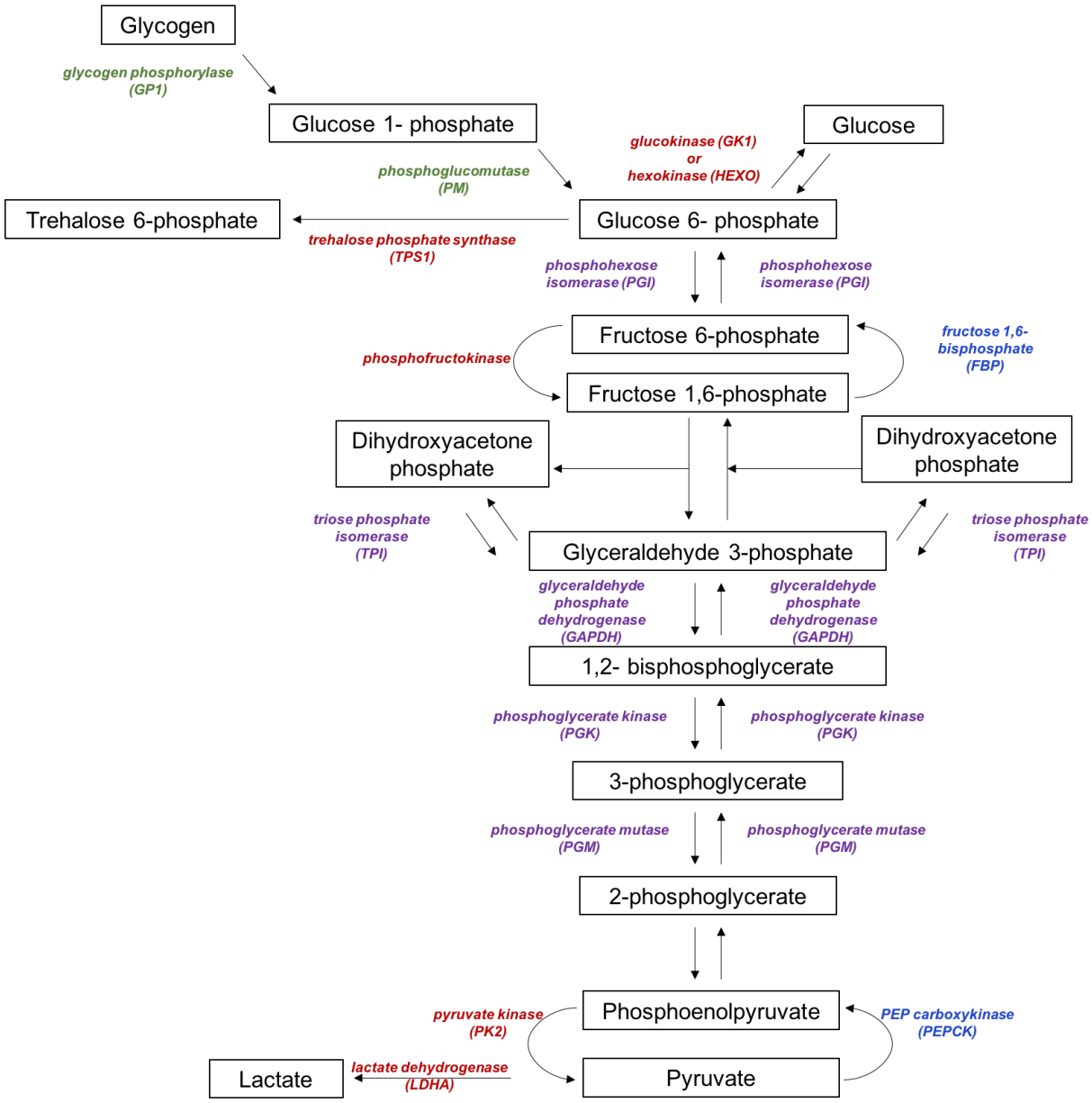


Figure 6: Glycolysis pathway. Glycolysis and gluconeogenesis metabolism and synthesis of glucose to pyruvate including glycolytic intermediates and enzymes.

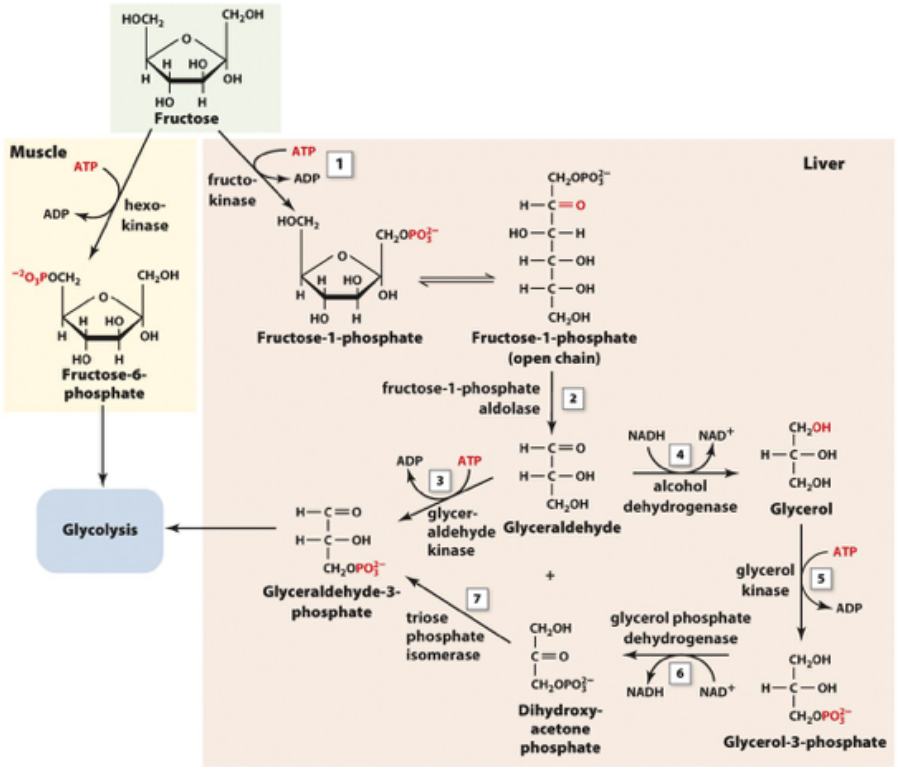


Figure 7: Metabolism of Fructose in the Liver and Muscle. Molecular mechanism for breakdown and conversion of fructose into the glycolysis pathway for both liver and muscle (Figure adapted from: Voet 2015).

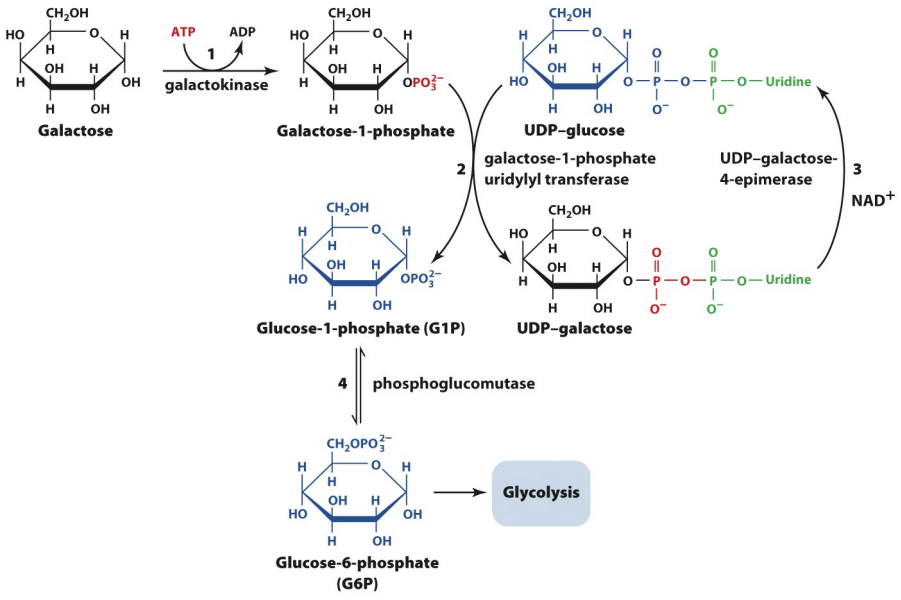


Figure 8: Metabolism of Galactose. Molecular breakdown and conversion of galactose into the glycolysis pathway (Figure adapted from: Voet 2015).

To maintain proper sugar levels within the body, other pathways are used aside from glycolysis. Gluconeogenesis is the process of synthesizing glucose from energy, allowing the body to maintain proper glucose levels. Glycogen synthesis is also used to maintain proper sugar level by storing excess glucose in the muscles and liver as glycogen. Glucose is converted to glycogen using glycogen phosphorylase and phosphoglucomutase (Figure 6) (Institute of Medicine, Food, and Nutrition Board, 2005).

When Colony Collapse Disorder (CCD) became a common occurrence, commercial beekeepers switched the bee's food from sucrose to a less expensive, but more processed substitute of high fructose corn syrup (HFCS). However, there might be a correlation between the types of food provided and CCD. Therefore, the goal of this project is to identify changes in carbohydrate metabolism due to HFCS. To determine these changes, gene expression was measured within the glycolysis and gluconeogenesis pathways after bees were exposed to a variety of sugar sources.

MATERIALS AND METHODS

Honeybee Exposure

To measure changes in metabolism an exposure was conducted. Bees were taken from 12 hives in August 2013 from Warm Colors Apiary in Deerfield, MA, and placed in eight oz. glass jars with a burlap cover. To avoid high amounts of isolation stress there were 5 bees per jar, a piece of empty comb (6x4 cm approx.), and a feeder with food, and a feeder with water. Bees were monitored twice a day for 7 days, and fed honey, sucrose, corn syrup (CS), or high fructose corn syrup 55 (HFCS) in complete darkness. At exposure termination bees were immediately euthanized and placed in 800 uL of Tri- Reagent LS (Molecular Research Center, Inc., Cincinnati, OH). Samples were stored in -80°C conditions until mRNA was extracted.

RNA Extraction

Bees were thawed on ice. When thawed, tissues were disrupted manually and homogenized 4 times for 4 seconds. Homogenized sample was left at room temperature for 5 minutes before addition of 200 uL of chloroform. Once added, tubes were shaken by hand vigorously for 15 seconds and left at room temperature for 2-3 minutes. All samples were centrifuged at 12,000g for 15 minutes at 4°C. The upper aqueous layer was transferred and 700 uL of 70% ethanol was added and mixed thoroughly. RNA extraction was completed using RNAeasy Mini Kit according to manufacturer's instructions (QIAGEN Sciences Inc., Germantown, VA). RNA concentrations were quantified using ThermoFisher Scientific Nanodrop 2000 (Thermofisher Scientific Inc., Cambridge, MA) and the samples stored at -80°C.

cDNA Synthesis

cDNA synthesis was prepared using reverse transcription enzyme solution. 2 ug of RNA was held at 65°C for 5 minutes. RNA was reverse transcribed in 25 uL of reverse transcription solution that included 15.525 uL of DEPC water, 400U of MMLV, 25U RNAsin, 0.65 mm dNTPs, and 0.2 uM random hexamers in the presence of 1X RT Buffer. 25 uL of the reverse transcription enzyme solution was then added to each sample and incubated at 37°C for 1 hour. Samples were frozen at -20°C upon completion. Samples were tested using a 2% gel electrophoresis with ethidium bromide and 1X SB Buffer, to ensure presence of cDNA. Solution with cDNA sample was made and run in PCR. Dye was added and samples were loaded with primer LDHA and DNA ladder into the wells. Gels were electrophoresed for 20 mins and then placed under UV light for analysis.

qPCR

To determine changes in gene expression, 14 genes were identified in the glycolysis pathway (Figure 6) from NCBI, along with 2 housekeeping genes, 18s rRNA and 28s rRNA. Primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). Standard curves were performed for all genes before running full plates. Full plates consisted of the samples for each treatment run in quadruplicate, for all 9 hives. Initial denature at 95°C for ten minutes was followed by 40 cycles of 95°C for 15 seconds, with an annealing of 62°C for 1 minute.

All primers followed this protocol except PEPCK which had an annealing temperature of 51°C. Successful standard curves had an R^2 of ≥ 0.990, efficiency of 90-110% and one peak for the melt curve. Gene plates were completed using Applied Biosystems QuantStudio 6 Flex Real-Time PCR, 384 well (qPCR) (Applied Biosystems, Framingham, MA), and included the gene being tested, individual samples from 9 hives in each treatment group, and a standard curve. Gene expression was measured within the three pathways by normalizing the target gene (Table 2) from a standard curve and dividing by the concentration of housekeeping genes 18s rRNA and 28s rRNA. A t-test using paired comparisons between honey and all other treatments was performed using Microsoft Excel.

Table 2: Primers Quantified using QPCR to Determine Changes in Gene Expression.

Pathway	Gene	Accession #	Primer L	Primer R
Glycolysis	GK1	NC_007080.3	TTGCAAGCTTTGATTCATGG	AGCAGCTCCATGTCGAAAAT
Glycolysis	PFK	NC_007079.3	TTGCAATGGCTGCTAAAATG	TCCTTGCTGCATATGACCTG
Glycolysis	LDHA	NC_007076.3	CAACGCGTTTGGATGTAATG	TGTTCAAGCAGAAACGAACG
Glycolysis	TPS1	NC_007075.3	GGAAAGGCACTTGAGCAGAC	TCTTGTCGAGGACACAGTCG
Glycolysis	PK2	NC_007073.3	TAGGGTGGAGCTGCTGAAGT	CATCGACAAAAACGCGACTA
Glycogen	PM	NC_007084.3	AATTGAACCAGTTGGGCAGA	GCGTCTCTGATTGCTTTTCC
Glycogen	GP	NC_007078.3	AAATGGAGGCTTGGGAAGAT	TTCCATAACGCAACCAATCA
Both pathways	TPI	NC_007085.3	AAAATCGGGTTGGAAGGAAC	CGACCCATGGCCTTAGAGTA
Both pathways	PGK	NC_007085.3	AAATGCAACTGTTGGTGCAG	GCAGGACCATTCCATACGAT
Both pathways	GAPDH	NC_007079.3	GGACAAAAGCTGGAGCTGAA	AAACAAACATGGGTGCATCA
Both pathways	PGM1	NC_007084.3	AAGTACCCACAGGCTGGAAA	CGCGAATATGATCTGAACCA
Both pathways	PGI	NC_007070.3	TAAGGCAAAAGCACGTGAAG	CAGCATTCACATCTGGCATTA
Gluconeogenesis	FBP	NC_007070.3	TGGAGATTGAAAAACGTGGA	CATGGCCAACCGAAAGTATT
Gluconeogenesis	PEPCK	NC_007072.3	TGGTGGAATCTTTTGGGAAG	CTGGGGAACAGAATCTGGAA
Housekeeping	18s rRNA	AB126807.1	CGCACGAGATTGAGCAATAA	GTACAAAGGGCAGGGACGTA
Housekeeping	28s rRNA	AB126808.2	GAGAGTGCAGCCCTAAGTGG	TCTCCCCATTCGATCTTTTG

RESULTS

After 7 days, the exposure experiment was completed with immediate euthanasia of all honeybees. RNA was successfully extracted for all 35 samples, and concentrations were determined by the 260/280 ratios on the Nanodrop (Table 3). Two of the samples were not used due to low RNA concentrations. RNA was synthesized into cDNA, and the presence of all cDNA was verified using gel electrophoresis (Figure 9).

All genes were tested using qPCR, and produced a standard curve with and R^2 between 0.980 and 0.999. Melt curves generated one peak indicating one product from amplification. A change in expression was observed for genes only in glycolysis when fed HFCS, and was not observed when bees were fed CS (Figure 10). Glucokinase (GK1), phosphofructokinase (PFK), trehalose-6-phosphate synthase (TPS1), pyruvate kinase (PK2), and lactate dehydrogenase (LDHA), all specific to the glycolysis pathway were down-regulated 45-55% in HFCS compared to honey. Additionally, genes converting glycogen into glycolysis such as phosphoglycerate mutase (PM) and glycogen phosphorylase (GP1) were also down-regulated 30-60% when HFCS compared to honey (Figure 11).

Although glycolysis-only genes are down-regulated, genes in both glycolysis and gluconeogenesis such as glyceraldehyde phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), triose phosphate isomerase (TPI), did not change in gene expression (Figure 13), with the exception of phosphoglucomutase (PGM1) and phosphohexose isomerase (PGI). PGI was decreased by 50% in HFCS when compared to honey. Genes only in gluconeogenesis such as fructose bisphosphate (FBP) and phosphoenolpyruvate carboxylase (PEPCK), also did not have down-regulation when HFCS was compared to honey (Figure 12).

When fed CS, 50% of the same genes only in glycolysis were down-regulated when compared to honey. A similar trend was also observed in sucrose. In addition to changes in HFCS, LDHA was down-regulated in CS by 60%, and PM 65% in sucrose (Figure 10). TPS1 and PK2 were affected in all treatments when compared to honey (Figure 10). TPS1 was down regulated 58% in CS and 55% in sucrose. PK2 was decreased by 55% in CS and 50% in sucrose. For genes only in gluconeogenesis, PEPCK was down-regulated in response to CS by 60% and sucrose 50%. For genes in both pathways, PGM1 was down-regulated in CS by 50%, and PGI 51% in CS and 65% in sucrose (Figure 13). No genes were up-regulated compared to honey when bees were fed HFCS or CS.

Table 3: Ratios and Concentrations of RNA in Exposure Bees after RNA extraction.

Treatment	ng/uL	260/280	260/230
Honey 1	2196.9	2.11	2.27
Honey 2	2386.6	2.12	2.33
Honey 3	2032.8	2.14	2.07
Honey 4	1613.1	2.07	2.07
Honey 5	2501.1	2.10	2.28
Honey 6	1826.6	2.06	2.17
Honey 10	2614.6	2.11	2.24
Honey 11	1176.0	2.11	2.16
Honey 12	1915.4	2.05	2.18
HFCS 1	1870.9	2.03	2.03
HFCS 2	2213.7	2.12	2.20
HFCS 3	1072.6	2.12	2.38
HFCS 4	2254.2	2.11	2.12
HFCS 5	2243.5	2.14	2.17
HFCS 6	1842.2	2.02	2.07
HFCS 10	1819.4	2.07	2.16
HFCS 11	1715.5	2.04	2.12
HFCS 12	2209.0	2.13	2.24
CS 1	2431.0	2.11	2.27
CS 2	1824.5	2.04	2.06
CS 3	2935.2	2.08	2.22
CS 4	886.4	2.12	2.13
CS 5	1553.6	2.09	2.19
CS 6	1720.5	2.08	2.08
CS 10	2857.3	2.06	2.19
CS 11	1468.6	2.11	2.19
CS 12	3470.8	1.94	1.99
Sucrose 1	2556.5	2.12	2.28
Sucrose 2	2301.5	2.11	2.26
Sucrose 3	1392.1	2.09	2.23
Sucrose 4	1200.7	2.13	2.16
Sucrose 5	2508.8	2.13	2.30
Sucrose 10	1643.8	2.08	2.02
Sucrose 11	2520.8	2.08	2.24
Sucrose 12	3318.7	1.99	2.07

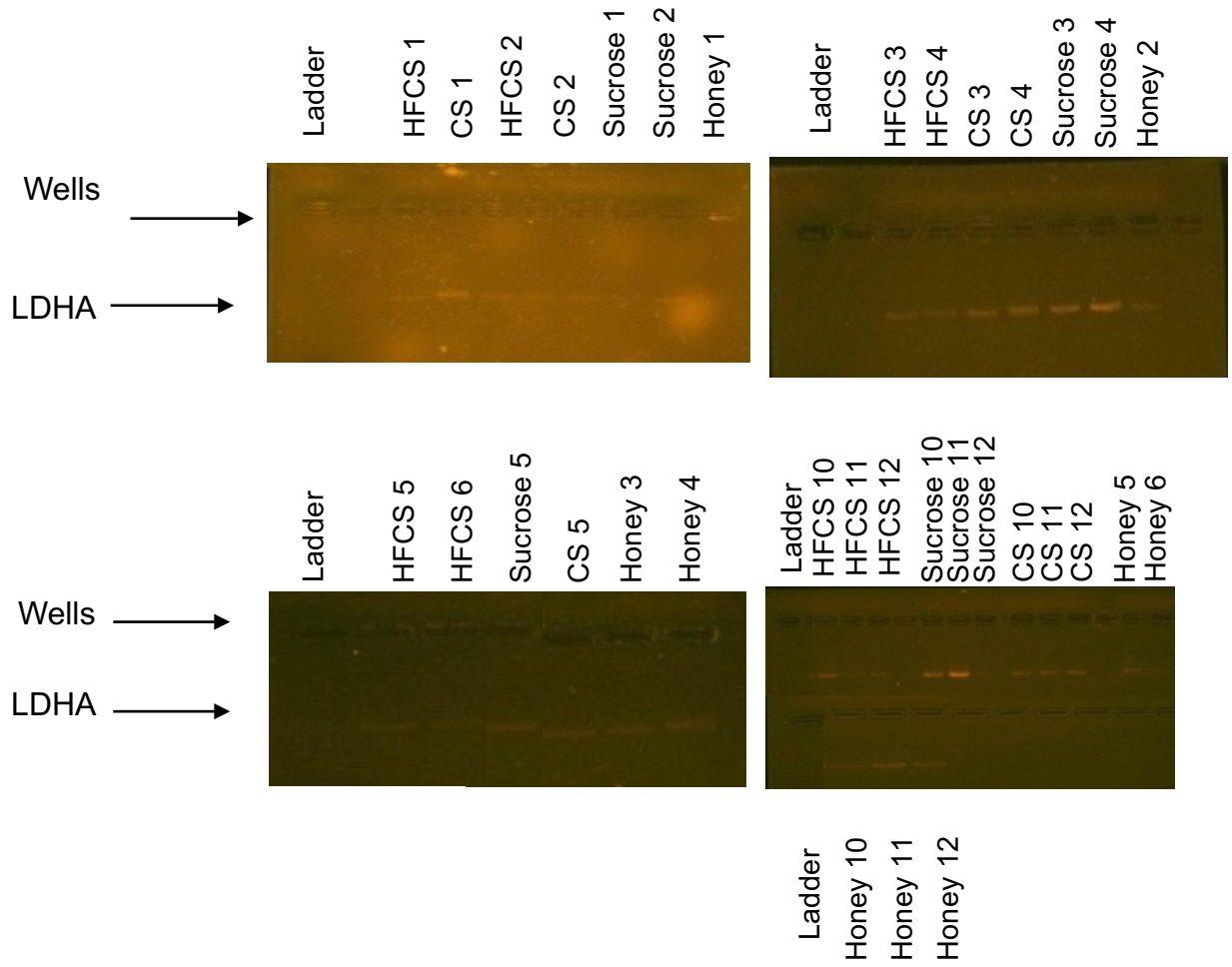


Figure 9: LDHA Amplification Determines Presence of cDNA. PCR amplification of LDHA verifies presence of cDNA in exposure treatment groups. Wells labeled by sugar group followed by hive number. All 2% gels were stained with ethidium bromide and electrophoresed for 20 minutes.

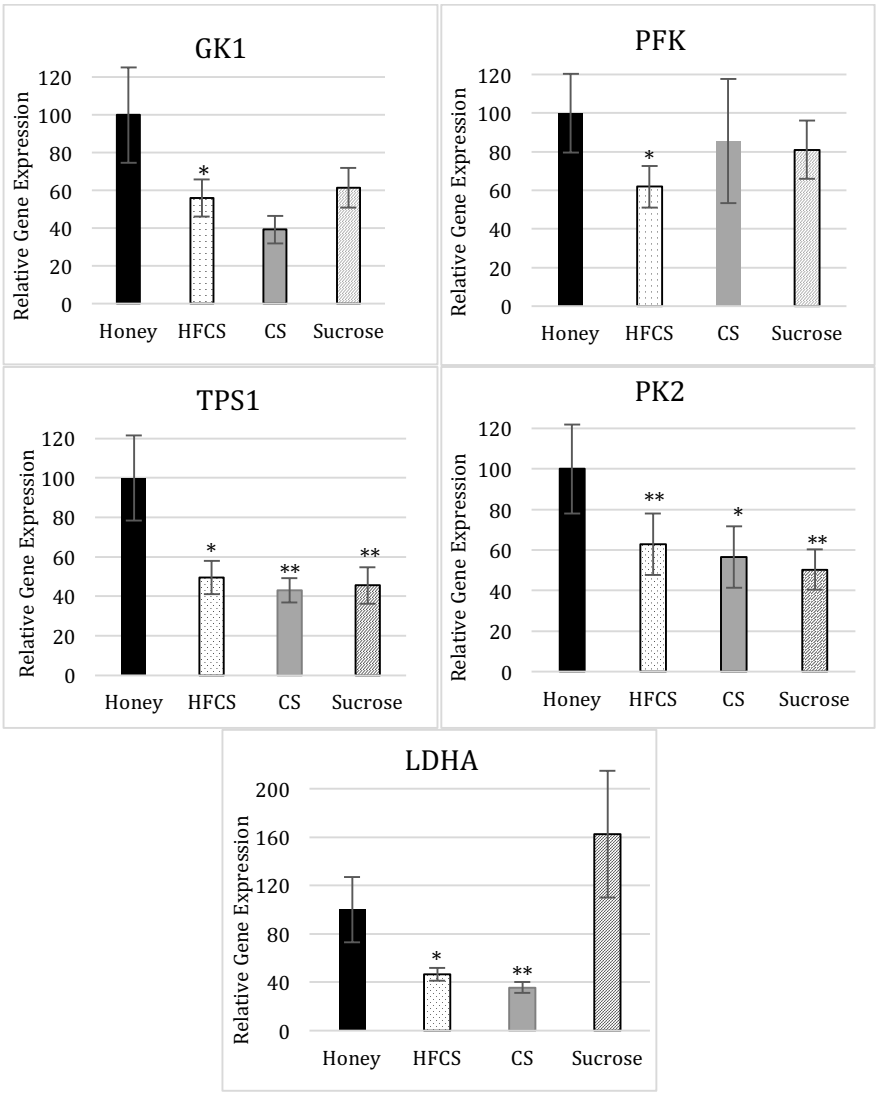


Figure 10: Genes in Glycolysis have down-regulation in expression for High Fructose Corn Syrup (HFCS) when compared to honey. Gene expression for GK1, PFK, TPS1, PM, PK2, and LDHA after qPCR analysis. All genes are down regulated in response to HFCS. Some genes in glycolysis have a similar change in expression when exposed to CS and sucrose. T-test using paired comparisons between honey and all other treatments. (** = $P \leq 0.05$, * = $P \leq 0.1$, n = 9) (mean $\pm$ St. Error)

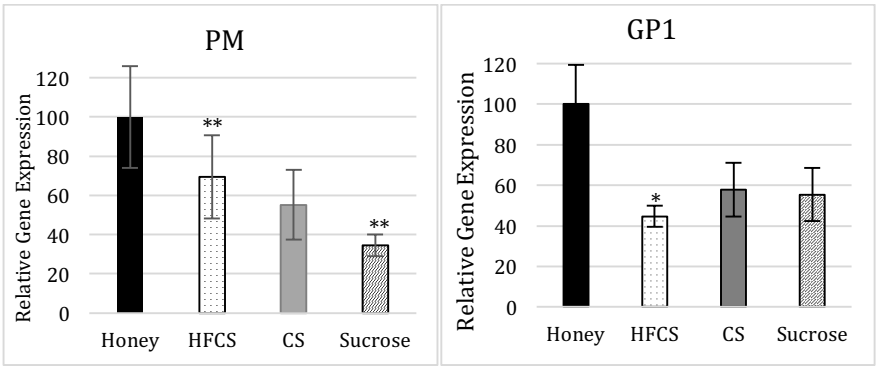


Figure 11: Down-regulation of Genes involved in Glycogen due to HFCS. Genes phosphoglucomutase (PM) and glycogen phosphorylase (GP1) are down regulated when exposed to HFCS compared to honey. T-test using paired comparisons between honey and all other treatments. (**=$P \leq 0.05$, * = $P \leq 0.1$, n =9) (mean $\pm$ St. Error)

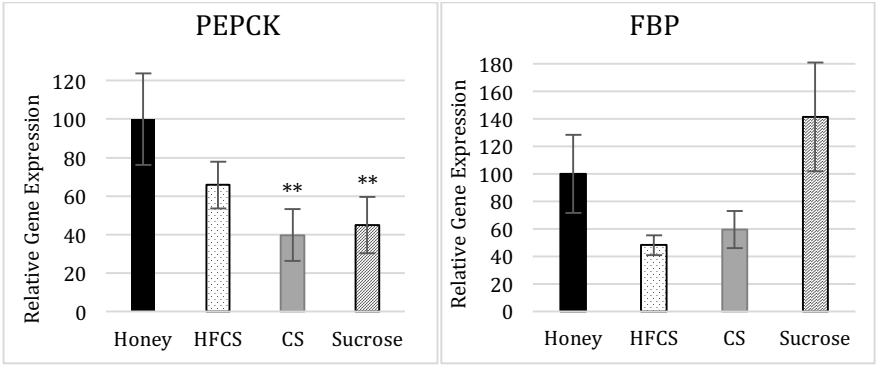


Figure 12: No down-regulation of genes only in gluconeogenesis and amino acid genes when exposed to High Fructose Corn Syrup (HFCS). Genes in just gluconeogenesis were not affected when bees were fed HFCS compared to honey. One gene in gluconeogenesis (PEPCK) and one gene in a phenylalanine amino acid pathway (H-1,2-D) had altered expression in CS when compared to honey. PEPCK also had down-regulation when exposed to Sucrose. FBP did not change in any treatment group. T-test using paired comparisons between honey and all other treatments. (**= $P \leq 0.05$, * = $P \leq 0.1$, n =9) (mean $\pm$ St. Error)

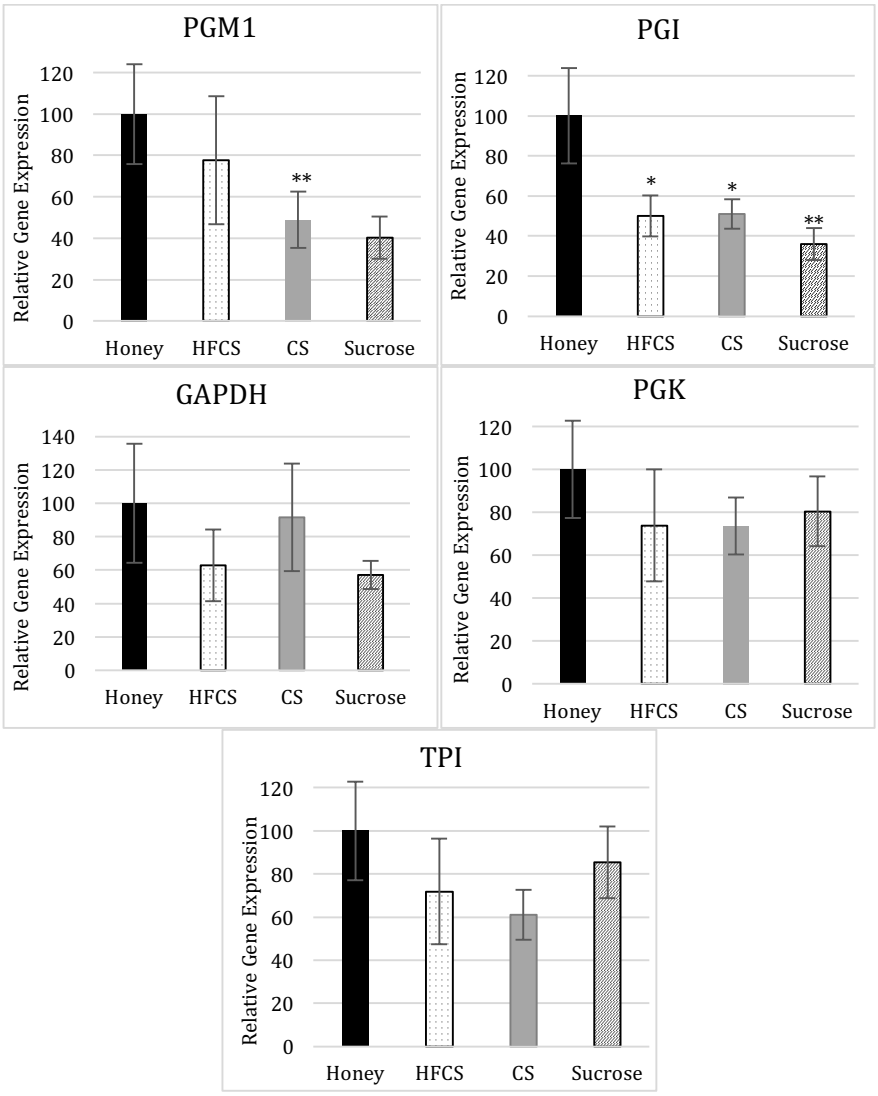


Figure 13: No down-regulation for genes in both glycolysis and gluconeogenesis when exposed to High Fructose Corn Syrup (HFCS). qPCR analysis of genes in both glycolysis and gluconeogenesis, with the exception of PGI and PGM, determines they do not exhibit changes in gene expression in HFCS and CS treatment groups when compared to honey. PGI also has a change in expression when exposed to Sucrose. GAPDH, PGK, and TPI did not change in any treatment group. T-test using paired comparisons between honey and all other treatments. (** = $P \leq 0.05$, * = $P \leq 0.1$, n =9) (mean $\pm$ St. Error).

DISCUSSION

HFCS is seen as an attractive energy resource for foraging honeybees due to its sweetness. In this study, however, HFCS down-regulated all genes involved in glycolysis when compared to honey (Figure 10), while CS and sucrose only effect 50% of the glycolysis genes. Down-regulation of glycolysis could result in lowered energy levels. When bees fly, glycogen in flight muscles is depleted. On average bees die after flying a total of 800 km, which can be spread out or condensed between 5-30 days. This is due to the breakdown in the mechanism that metabolizes carbohydrates into glycogen (Neukirch 1982). Over time, the mechanism that breaks down glycogen gradually loses efficiency and fails. Once the mechanism breaks down, it leaves workers unable to synthesize more glycogen for flight. If honeybees were given a consistent and insufficient carbohydrate source, glycogen reserves could already be limited due to the majority of nutrients going straight to the liver (Figure 14). Glycogen phosphorylase (GP1), which converts glycogen into glucose 1-phosphate and phosphoglucomutase (PM), which converts glucose-1-phosphate to glucose-6-phosphate were both down-regulated in bees who were fed HFCS. With HFCS having high fructose concentrations and small glucose concentrations, only a small percentage of a HFCS diet would be delivered to other parts of the body as energy. This could be contributing to Colony Collapse Disorder (CCD) by causing their glycogen mechanism to fail more quickly before they can return to the hive for nutrients.

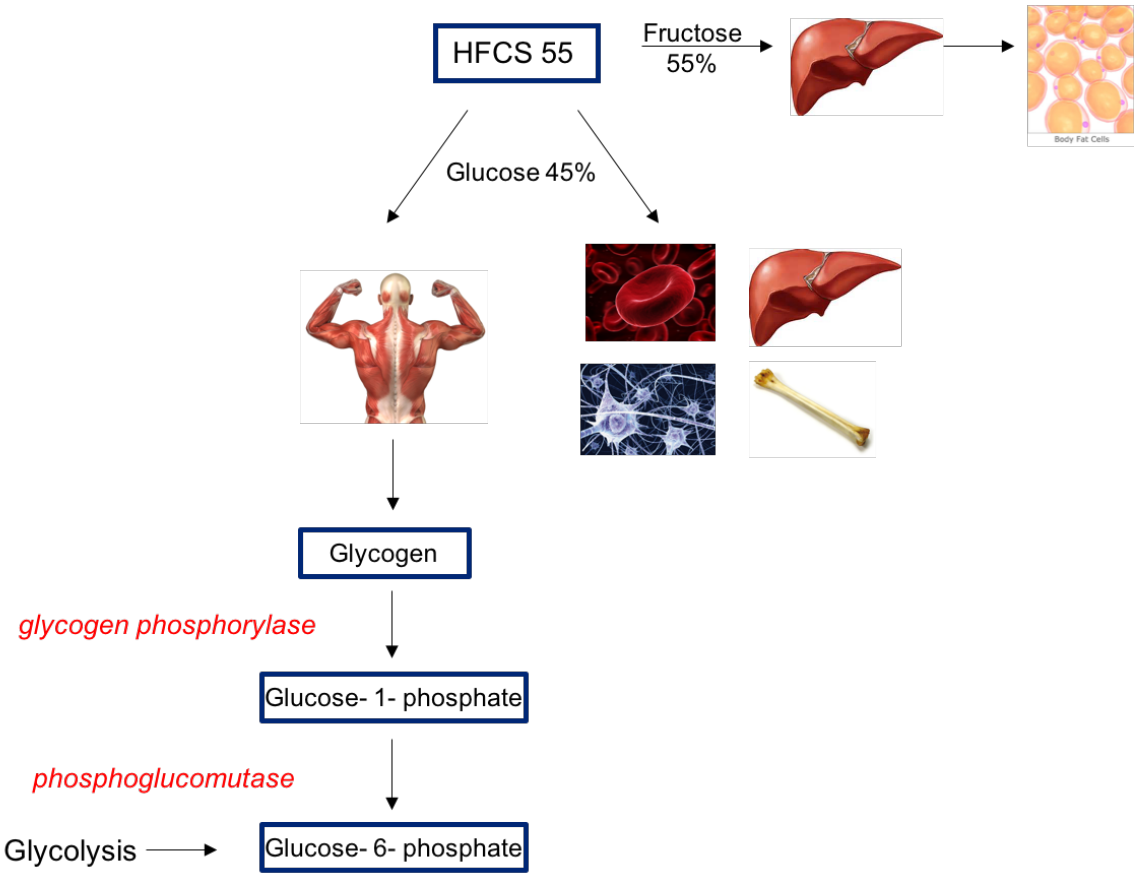


Figure 14: Tissue use of High Fructose Corn Syrup (HFCS) 55 throughout the body.

Although honey and HFCS have similar glucose and fructose percentages (Table 1), honey consists of a sugar called trehalose that is not present in HFCS. Trehalose is the main sugar found in the hemolymph of honeybees, reaching concentrations between 2–40 mg/mL (Blatt, J. and Roces, F. 2001). Trehalose is synthesized using glucose, and is distributed throughout the body or stored as glycogen (Zoltowaska et al., 2011). Glucose and fructose are also found in hemolymph, although exist in very low amounts (15 ug/mL for glucose, 7 ug/mL for fructose) (Leta et al., 1996). With HFCS not containing trehalose, the honeybee relies on extremely low hemolymph sugar levels to distribute energy to the rest of the body. This may be causing the down-regulation seen in genes involved in glycolysis, as well as the down-regulation of glycogen phosphorylase and trehalose phosphate synthase (Figure 10, 11). Both genes being only in glycolysis, are involved in the production of trehalose and breakdown of glycogen.

Increased sweetness of artificial syrup dishes may also be seen as favorable by beekeepers. Honeybees were documented to prefer artificial syrup dishes compared to regular nectar after being monitored in a study. Bees were found to make 150 trips per day to artificial syrup dishes (Butler et al., 1943), but only 110 flights per day for water (Park, 1928b). It was also noticed when clusters of honeybees were found at HFCS plants feeding on HFCS spills that occurred during loading (Barker and Lehner, 1978).

To further demonstrate that honeybees have a preference to HFCS a behavioral assay will need to be conducted. A T-shaped chamber will be constructed out of acrylic tubing, with HFCS and another sugar located at the ends. Bees will be released into the chamber and monitored on which sugar they choose (Figure 15). A learning assay will also be conducted using the same apparatus, with food only one side and no food on the other. Bees will be monitored on how fast they can find the sugar source, and time will be recorded (Figure 16). Different bees will be used for each study.

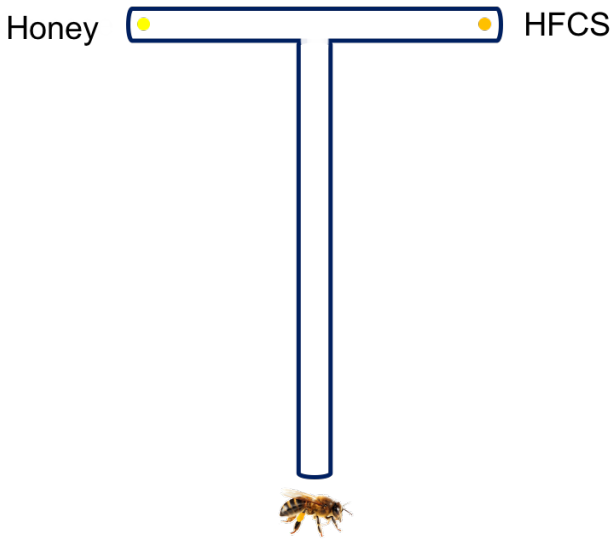


Figure 15: Sugar Preference Assay for HFCS. Bees will be released within a T-shaped apparatus to test behavioral preference of HFCS compared to other sugar treatments. Sugar sources will be located at the ends of the chamber and bees will be released from the bottom.

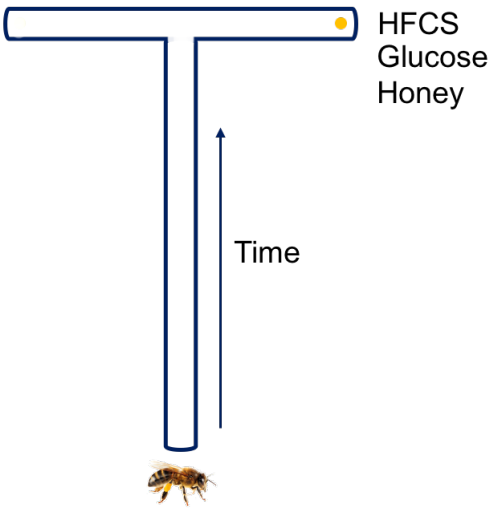


Figure 16: Learning Assay for HFCS. Bees will be released within a T-shaped apparatus to test for learning capacity when given one type of sugar. Bees will be released from the bottom of the T-shaped apparatus, and timed to see how fast the sugar source can be located. Times will be compared between treatment groups.

With HFCS down-regulating glycolysis and bees preferring artificial syrup dishes to natural sources, less glycogen could be stored due to decreased productivity in glycolysis. In addition, it has been recorded that there has been decreased longevity in worker bees given HFCS when compared to honey (Barker and Lehner, 1978). Decreased longevity could be caused by hydroxmethylfurfural (HMF), a compound that is formed when fructose is dehydrated at high temperatures. Forming at temperatures above 45°C, HMF is toxic to bees, causing dysentery-like symptoms (Parker et al., 2010). Although thermoregulation in the hive is tightly monitored, ranging from 33.5°C-37.0°C (Fahrenholz et al., 1989), weak or sick colonies could not perform thermoregulation as efficiently. High amounts of HMF could be a threat to commercial beekeepers due to the location of food source in man-made hives. At the top of the hive, sugar sources are in a tight, sealed space under a metal cover. The sugar is readily accessible by bees from the inside. If fed HFCS, it could be rapidly heated due to being under sunlight or from internal temperatures of the hive, causing toxic HMF to be released.

Honeybees, as well as other bees, pollinate a wide variety of plants, flowers, and crops (Table 4). They offer a pollination service that has a large economic impact on humans, valued to be worth 3 billion in the United States. Without their help to maintain a proper balance in our ecosystem, our forest, parks, meadows, and crops will cease to survive (US Fish and Wildlife 2017). If HFCS is changing glycolysis in honeybees (Figure 10), it should also inform a change in how commercial beekeepers feed their hives.

Table 4: Honeybee dependent crops (Sarich, 2013).

Raspberries	Apples	Mangos	Cauliflower
Pears	Starfruit	Broccoli	Grapes
Avocado	Cabbage	Strawberries	Cactus
Pomegranate	Coconut	Watermelon	Tomatoes
Green peppers	Red peppers	Chili peppers	Limes
Buckwheat	Coffee	Cotton	Vanilla

An insufficient food source such as HFCS is decreasing glycolysis efficiency, altering the amount of ATP made for oxidative phosphorylation. This could be effecting overall energy levels in colonies, decreasing glycogen storage in flight muscle, and potentially contributing to CCD.

Unlike honeybees that are commercialized and controlled due to their high economic value, populations of other pollinators in nature are not regularly monitored. HFCS can attract and affect pollinators such as monarch butterflies or bumble bees, causing large environmental impacts. Once seen flourishing in over 28 states, the rusty patch bumble bee (*Bombus affinis*) were once common sight from Connecticut to South Dakota. Although in early 2017, the United States Fish and Wildlife Service listed the rusty patch bumble bee as the first bee in U.S history to be listed as endangered. Over the past 20 years, the bumble bee population has plummeted 87% since the late 1990s. It now only exists scattered across 13 states and one province (US Fish and Wildlife 2017). If honeybees were to suffer a similar fate as the rusty patch bumble bee, it would cause a severe agricultural decline and large threat to food security.

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